

Coordinated regulation of apical hook development by gibberellins and ethylene in etiolated *Arabidopsis* seedlings

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Dark-grown *Arabidopsis* seedlings develop an apical hook when germinating in soil, which protects the cotyledons and apical meristematic tissues when protruding through the soil. Several hormones are reported to distinctly modulate this process. Previous studies have shown that ethylene and gibberellins (GAs) coordinately regulate the hook development, although the underlying molecular mechanism is largely unknown. Here we showed that GA₃ enhanced while paclobutrazol repressed ethylene- and EIN3-overexpression (*EIN3ox*)-induced hook curvature, and *della* mutant exhibited exaggerated hook curvature, which required an intact ethylene signaling pathway. Genetic study revealed that GA-enhanced hook development was dependent on HOOKLESS 1 (HLS1), a central regulator mediating the input of the multiple signaling pathways during apical hook development. We further found that GA₃ induced (and DELLA proteins repressed) *HLS1* expression in an ETHYLENE INSENSITIVE 3/EIN3-LIKE 1 (EIN3/EIL1)-dependent manner, whereby EIN3/EIL1 activated *HLS1* transcription by directly binding to its promoter. Additionally, DELLA proteins were found to interact with the DNA-binding domains of EIN3/EIL1 and repress EIN3/EIL1-regulated *HLS1* expression. Treatment with naphthylphthalamic acid, a polar auxin transport inhibitor, repressed the constitutively exaggerated hook curvature of *EIN3ox* line and *della* mutant, supporting that auxin functions downstream of the ethylene and GA pathways in hook development. Taken together, our results identify EIN3/EIL1 as a new class of DELLA-associated transcription factors and demonstrate that GA promotes apical hook formation in cooperation with ethylene partly by inducing the expression of *HLS1* via derepression of EIN3/EIL1 functions.

Keywords: gibberellins; ethylene; EIN3/EIL1; HLS1; apical hook

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Introduction

Sessile plants cannot move, so they can only alter their growth pattern through differential growth to adapt to the changing environment. In dicotyledonous plants, the first process of differential growth during their lifespan is the formation of an apical hook, which protects cotyledons and apical meristematic tissues from mechanical damage when elongating seedlings protrude through the soil to the surface [1]. The apical hook formation in *Arabidopsis*

is caused by differential cell growth on the opposite side of the hypocotyl, where the rates of cell elongation in the outside are faster than the inner side [2, 3]. This process is coordinated by several hormones. Auxin has been extensively studied for its role in apical hook development. Exogenous application of auxin [4] or genetic manipulation of auxin-synthesis genes [5-7] causes defects in apical hook formation. Moreover, blocking polar auxin transport by treatment with naphthylphthalamic acid (NPA) prevents hook formation [4, 8]. An asymmetrical accumulation of auxin is thought to be necessary for the differential cell growth during hook development [8].

Besides the regulation by auxin, ethylene is another regulator of hook development. Exogenous treatment with ethylene or its biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) leads to the development of exaggerated apical hook, together with a short,

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thickened root and hypocotyl, which is known as the “triple response” of etiolated seedlings [9, 10]. A number of ethylene response mutants that show an aberrant triple response phenotype have been identified in *Arabidopsis*. Of these mutants, the etiolated seedlings of ethylene-insensitive mutants, such as *etr1* [11], *ein2* [12], and *ein3* [13], exhibit reduced apical hook, long hypocotyls and roots even in the presence of ethylene. In contrast, mutants resulting from either ethylene overproduction (*eto1*) [12] or activated ethylene signaling (*ctr1*) [14] show constitutively exaggerated apical hook. Mutations in *HOOKLESS 1 (HLS1)*, which encodes a protein with sequence similar to *N*-acetyltransferase, have also been identified that exhibit a complete loss of hook formation in the presence of exogenous ethylene application [8, 12]. While the *hls1* mutant fully suppresses the phenotype of exaggerated hook curvature of *ctr1* or *eto1* [15], overexpression of *HLS1* results in a constitutive hook curvature [8], indicating an essential role of *HLS1* in the regulation of hook development.

Multiple hormone interactions have been revealed in the control of apical hook development, in which the cross-talk between auxin and ethylene was extensively studied. Ethylene was reported to activate the transcription of *HLS1*, whose activity is required for the normal expression of auxin-responsive genes and the reporter gene *DR5::GUS* in the hook region [3, 8]. Moreover, a genetic screen has identified *AUXIN RESPONSE FACTOR 2 (ARF2)*, whose mutation suppresses the *hls1* phenotype. Ethylene downregulates the level of the *ARF2* protein in a *HLS1*-dependent manner [3]. These studies pinpoint *HLS1* as a key mediator of ethylene and auxin signaling in the regulation of the apical hook curvature. Meanwhile, two recent studies revealed direct regulation of the auxin polar transport machinery by ethylene through analyzing hook development kinetics [16, 17]. They found that ethylene induces exaggerated hook curvature by activating the transcription of an auxin influx carrier *AUX1*, as well as by altering *AUX1* protein distribution. Additionally, ethylene regulation of apical hook development also involves modulation of the activity of the PIN-dependent auxin efflux machinery through transcriptional and posttranscriptional mechanisms.

Another plant hormone gibberellins (GAs) were also reported to regulate the hook curvature. GA deficiency (*gal-t*) or treatment with paclobutrazol (PAC), an inhibitor of GA biosynthesis, inhibits ethylene-induced hook curvature of dark-grown seedlings [18], which is reversed by mutations resulting in defects in DELLAs [18]. DELLA proteins are key repressors of GA responses by inhibiting GA-regulated gene expression [19]. These repressors accumulate in the nucleus and

are rapidly degraded via the 26S proteasome pathway in response to GAs [20, 21]. Mutations within the DELLA domains make these proteins resistant to degradation and result in a GA-insensitive dwarf phenotype [22, 23]. It is thought that the DELLA repressors act negatively in GA responses by association with diverse transcription factors or regulators [24, 25], including the bHLH-family transcription factors PIF3 and PIF4 [26, 27], another bHLH protein ALCATRAZ (ALC) [28], and a GRAS family member SCL3 [29, 30]. In addition, DELLA promotes jasmonic acid signaling by directly binding to and sequestering jasmonic acid-signaling repressors, JAZ proteins [31]. Accumulating evidence has demonstrated that DELLA proteins act as central regulators that control a wide array of plant growth and development processes. The identification of additional associated transcription factors would shed light on the distinct actions of DELLA proteins in various GA-regulated responses.

Here we show that ethylene and GAs coordinately enhance apical hook formation by inducing the expression of *HLS1* in an ETHYLENE INSENSITIVE 3/EIN3-LIKE 1 (EIN3/EIL1)-dependent manner. In addition, we find that *HLS1* is a direct target gene of EIN3/EIL1, and that DELLA proteins inhibit the function of EIN3/EIL1 by associating with their DNA-binding domains. Therefore, our study provides new insight into the molecular mechanisms underlying the synergistic regulation of apical hook development by plant hormones.

Results

GA₃ enhances while PAC represses ethylene- and EIN3-induced hook curvature

Previous studies have shown that inhibition of GA biosynthesis by PAC prevents apical hook formation in both wild-type plants and the constitutive ethylene response mutant *ctr1-1* [18]. To further reveal the molecular details of this effect, we examined the hook phenotypes in other ethylene mutants upon GA₃ and/or PAC treatment. As shown in Figure 1A, PAC treatment inhibited the hook development of 3-day-old wild-type etiolated seedlings grown on Murashige and Skoog (MS) medium supplemented with or without ACC. In contrast, treatment with GA₃ reversed the PAC effect, and the hook curvature was even more exaggerated in the presence of ACC + PAC + GA₃ than that grown on ACC medium (Figure 1A). We further found that GA₃ treatment showed a more pronounced effect to enhance ACC-induced hook curvature in 6-day-old etiolated seedlings, as seedlings grown on ACC + PAC + GA₃ medium still exhibited exaggerated hook curvature, while only modest hook-bending was observed in seedlings grown on ACC

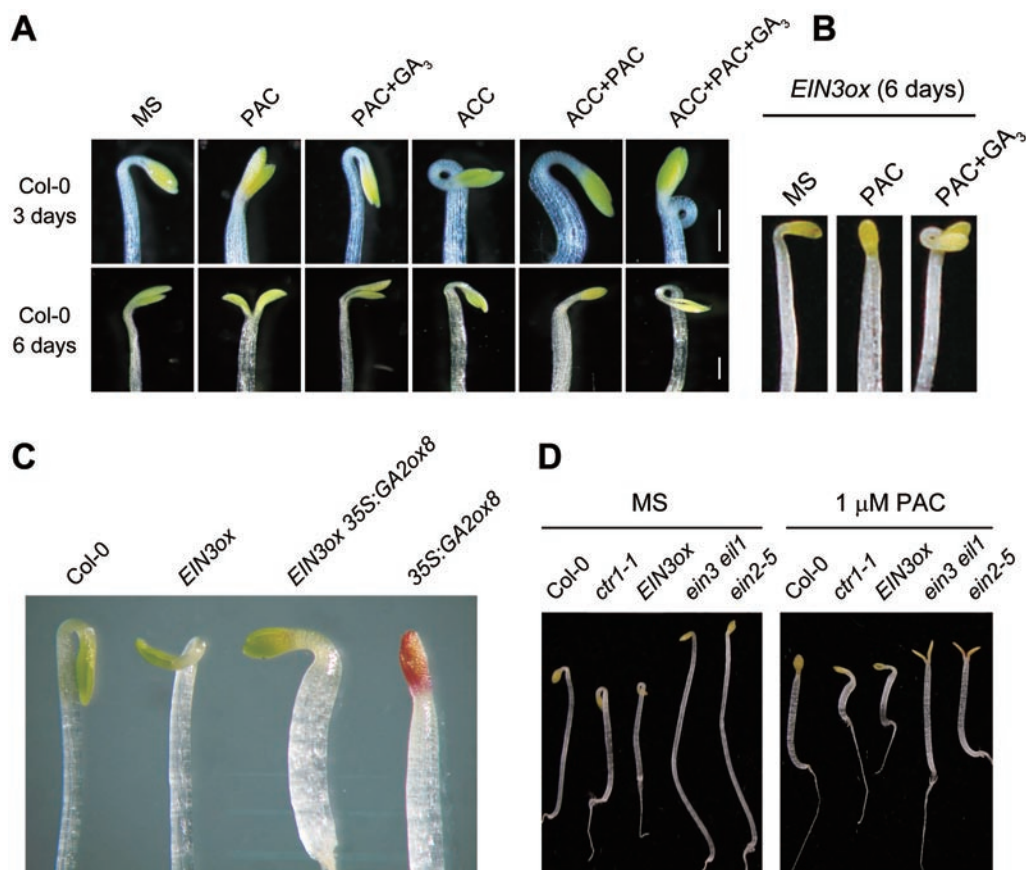


Figure 1 PAC represses while GA₃ enhances the ethylene-induced hook curvature. **(A)** The hook phenotype of 3-day-old (top) and 6-day-old (bottom) etiolated wild-type seedlings grown on the indicated medium (1 μM PAC, 10 μM ACC, and/or 10 μM GA₃). The scale bars represent 1 mm. **(B)** The hook phenotype of 6-day-old etiolated seedlings of *EIN3ox* grown on the indicated medium (1 μM PAC and/or 10 μM GA₃). **(C)** The hook phenotype of 3-day-old etiolated seedlings of indicated genotypes grown on MS medium. **(D)** The hook phenotype of etiolated ethylene-related mutants and transgenic plants grown on MS medium supplemented with or without 1 μM PAC for 3 days.

or PAC + GA₃ medium (Figure 1A).

EIN3/EIL1 are the central transcription factors in ethylene signaling [32], and overexpression of *EIN3* or *EIL1* results in constitutively exaggerated hook curvature [33, 34]. We next sought to examine whether GA or PAC treatment affects the hook phenotype of *EIN3* overexpression (*EIN3ox*). We found that PAC greatly suppressed the hook formation of 6-day-old *EIN3ox* seedlings, whereas GA₃ reversed the PAC effect, and further exaggerated the hook bending (Figure 1B). Consistent with the results from exogenous PAC treatment, promotion of GA decay through overexpression of *GA2ox8* [35, 36], which encodes a GA-inactivating enzyme (GA 2-oxidase), also repressed the hook curvature of *EIN3ox* (Figure 1C). These results indicate that application with GAs enhances while deprivation of GAs (by PAC treatment) represses *EIN3*-induced hook formation.

In addition, we found that upon PAC treatment, the 3-day-old seedlings with an activated ethylene signaling pathway (such as *ctr1* or *EIN3ox*) still showed bended hooks, whereas wild-type seedlings exhibited almost straight hooks, and ethylene-insensitive mutants (*ein3 eil1* or *ein2*) were completely hookless with opened cotyledons (Figure 1D). These results suggest that seedlings with constitutively activated ethylene signaling are partially resistant to PAC's repression of hook bending, whereas ethylene-insensitive mutants are hypersensitive to the PAC effect.

della mutant exhibits exaggerated hook curvature that requires an intact ethylene signaling pathway

DELLA proteins are critical repressors of GA responses [37]. To further determine how GAs and PAC affect ethylene-induced hook curvature, we analyzed the

hook phenotypes of various DELLA-related mutants and transgenic plants. The *della* quintuple mutant (with all five DELLA proteins mutated) [27] exhibited exaggerated apical hook on MS medium (Figure 2), which can be further enhanced by ACC treatment (Supplementary information, Figure S1). In contrast, transgenic plants overexpressing stabilized DELLA proteins (*35S:TAP-GAIΔ17* or *35S:TAP-RGAΔ17*) [27] exhibited decreased hook curvature compared with wild type, and this was particularly true for *35S:TAP-RGAΔ17*, which was almost hookless and evidently resistant to ACC treatment (Supplementary information, Figure S1). Together, these results suggest that DELLA proteins are repressors of ethylene-induced hook curvature, and that GA enhances ethylene action probably by destroying DELLA proteins.

To explore whether GA-enhanced hook curvature is caused by altered ethylene synthesis, *della*, *penta* (*gai-3*

gai-6 rga-t2 rgl1-1 rgl2-1), *EIN3ox*, *ctr1*, and ethylene-overproduction mutants (*eto1*) were grown on AVG medium, which blocks ethylene biosynthesis. As expected, AVG treatment repressed the constitutive hook curvature of *eto1* (Figure 2A), an ethylene-overproduction mutant [12]. However, it had no effect on *EIN3ox*, *ctr1*, *della*, or *penta* mutant (Figure 2A). These results indicate that the exaggerated hook curvature of *della* is not caused by increased ethylene biosynthesis.

However, we found that genetic mutations in the downstream ethylene signaling components (such as *ein2* and *ein3 eil1*) largely suppressed the exaggerated hook-curvature phenotype of *della* (Figure 2B). In addition, we observed that the *della* mutant exhibited an epinastic leaf phenotype, which results from exaggerated differential growth on the adaxial and abaxial sides of petioles [38]. Interestingly, the epinastic leaf phenotype of *della* was also suppressed by *ein2* or *ein3 eil1* (Supplementary information, Figure S2). Collectively, we conclude that EIN3/EIL1 are essential components required for the enhanced differential growth observed in *della*, including constitutive hook curvature and leaf epinastic growth.

GA-enhanced hook curvature is HLS1 dependent

HLS1 is essential for apical hook formation, and *hls1* fails to form a hook even in the presence of ACC [8, 12]. In order to investigate whether HLS1 is involved in GA/ethylene regulation of hook development, we observed the hook phenotype of *hls1* under GA and/or ACC conditions and found that none of these treatments induced hook formation in *hls1* (Figure 3A). Furthermore, the exaggerated hook curvature of *della* was fully suppressed by *hls1*, as the *hls1 della* mutant was also completely hookless (Figure 3B). These results suggest that GA- and DELLA-regulated hook development is dependent on HLS1.

GAs and ethylene induce HLS1 expression in an EIN3/EIL1-dependent manner

Since ethylene likely augments hook curvature by inducing *HLS1* expression, we asked whether GAs also regulate *HLS1* expression as ethylene does. Given the nearly saturated GA response in dark-grown seedlings [39], wild-type seeds germinated in dark for 24 h were first transferred to 0.2 μM PAC for 48 h to decrease the endogenous GA levels and then treated with 100 μM GA₃ for different time periods. We found that GA₃ treatment evidently induced *HLS1* expression after 2 h (Figure 4A). By demonstrating that the ethylene-induced expression of *HLS1* is EIN3/EIL1-dependent, as ACC treatment failed to induce *HLS1* expression in *ein3 eil1* (Figure 4B), we asked whether the GA₃-induced *HLS1*

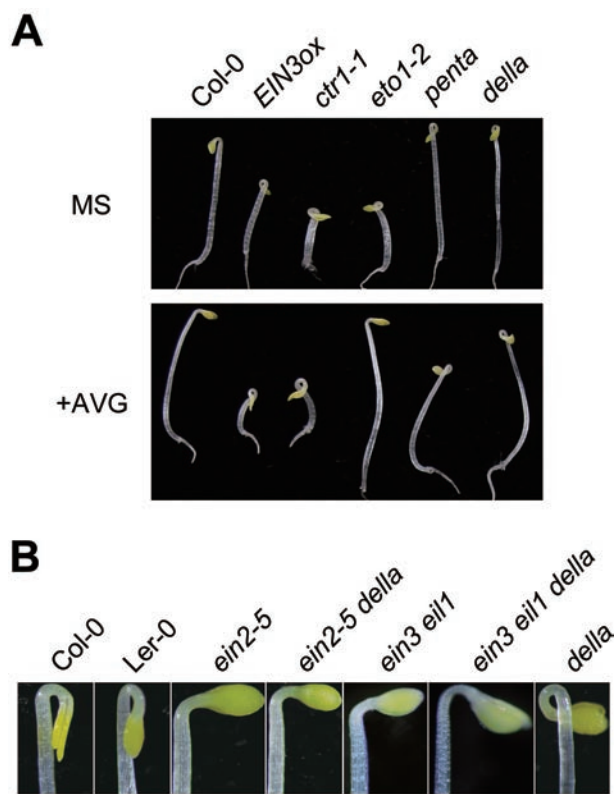


Figure 2 The *della* mutant exhibits exaggerated hook curvature that can be suppressed by *ein2* or *ein3 eil1*. **(A)** The exaggerated hook curvatures of *della* (with all the five DELLA-mutated) and *penta* (*gai-3 gai-6 rga-t2 rgl1-1 rgl2-1*) were not affected by AVG (ethylene biosynthesis inhibitor) treatment. Etiolated seedlings were grown on MS medium supplemented with or without 10 μM AVG for 3 days. **(B)** *ein2* or *ein3 eil1* suppressed the exaggerated hook curvature of *della*. Etiolated seedlings of indicated genotypes were grown on MS medium for 3 days.

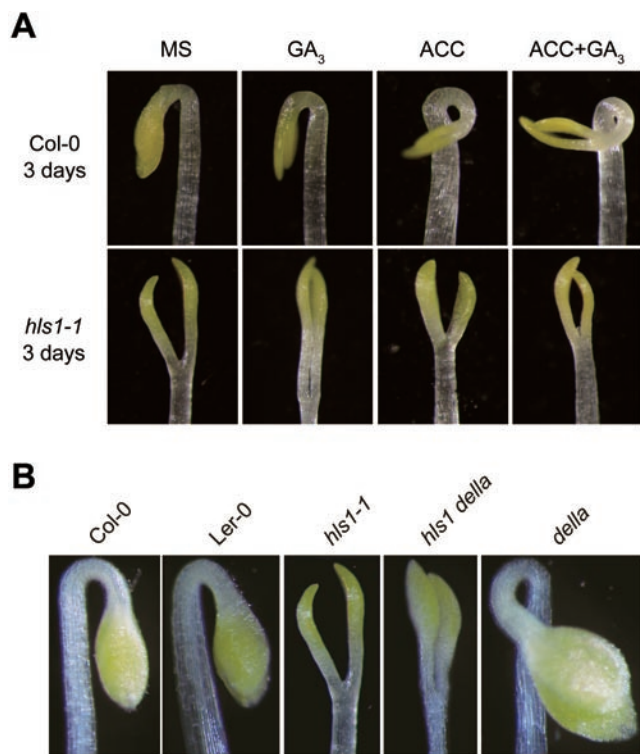


Figure 3 HLS1 is required for GA- and ethylene-induced hook curvature. **(A)** GA₃ and/or ACC treatment did not affect the hookless phenotype of *hls1*. Hooks of 3-day-old etiolated seedlings grown on the indicated medium were shown. **(B)** *hls1* prevented the hook curvature of *della*. Hooks of 3-day-old etiolated seedlings grown MS medium were shown.

expression requires EIN3/EIL1. Our results revealed that GA₃ also failed to induce *HLS1* expression in *ein3 eil1* (Figure 4A), indicating an essential role of EIN3/EIL1 in the *HLS1* induction.

Next, we examined *HLS1* expression in various GA-related mutants. *della* mutants showed dramatically higher *HLS1* expression than *35S:GA2ox8* transgenic plants (Figure 4C), which were presumed to accumulate less GAs and more DELLAs [36]. *spy-3*, another constitutive GA-response mutant with less DELLA accumulation [40], also showed higher *HLS1* expression similar to *della* (Figure 4C). Furthermore, we found that overexpression of EIN3 in the *ein3 eil1* background (*EIN3ox/ein3 eil1*) recovered the ethylene induction of *HLS1* expression (Figure 4B), supporting that EIN3 is sufficient for *HLS1* induction. Interestingly, manipulation of DELLA accumulation markedly influenced the effect of EIN3 on *HLS1* induction, as *35S:GA2ox8* greatly inhibited while *spy-3* evidently enhanced *HLS1* expression in the *EIN3ox* background (Figure 4C). Collectively, these results demonstrate that ethylene and GA₃ induce *HLS1* expression

in an EIN3/EIL1-dependent manner.

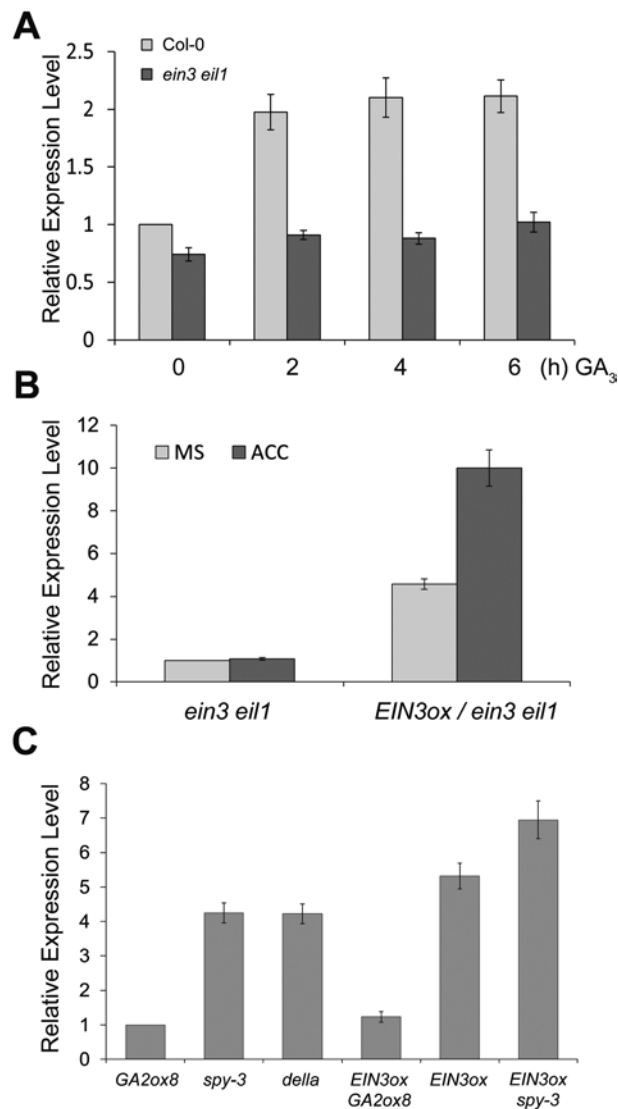


Figure 4 The GA₃- and ethylene-induced *HLS1* expression is EIN3/EIL1 dependent. **(A)** Quantitative reverse transcription PCR (qRT-PCR) analysis of the GA₃-induced *HLS1* expression in wild-type and *ein3 eil1* seedlings. Seeds germinating in dark for 24 h were transferred to 0.2 μM PAC for 48 h and then treated with 100 μM GA₃ for indicated hours prior to RNA extraction for qRT-PCR. **(B)** qRT-PCR analysis of the ethylene-induced *HLS1* expression in *ein3 eil1* and *EIN3ox/ein3 eil1* (overexpression of *EIN3* in *ein3 eil1*) seedlings. 3-day-old etiolated seedlings grown on MS medium were treated with or without 100 μM ACC for 4 h. **(C)** qRT-PCR analysis of *HLS1* expression in the indicated genotypes. 3-day-old etiolated seedlings grown on MS medium were used for RNA extraction. The *HLS1* expression levels in **A**, **B**, and **C** were normalized with the levels of β-tubulin. Mean ± SD, *n* = 3. All experiments were repeated at least twice with similar results.

EIN3 induces *HLS1* expression by directly binding to its promoter

We next investigated how EIN3/EIL1 induces the expression of *HLS1*. We generated a transgenic plant, which inducibly overexpressed EIN3-3FLAG in the *ein3 eil1* background (*iE/ein3 eil1*). After induction with 10 μ M estradiol, *iE/ein3 eil1* displayed exaggerated hook curvature compared with *ein3 eil1* (Supplementary information, Figure S3), confirming the sufficiency of EIN3 for augmenting hook curvature. We further found that estradiol treatment rapidly induced *HLS1* expression, within 1 h of EIN3 induction (Figure 5A), suggesting *HLS1* as a direct target gene of EIN3. A bioinformatic analysis found that a putative EIN3-binding site (EBS,

5'-ATTTCAA-3' [41]) is present in the -900 bp region upstream of the start codon of *HLS1* (Figure 5B). To determine whether EIN3 directly binds to the putative EBS, we performed electrophoretic mobility shift assays (EMSA) and found that EIN3 specifically bound to the wild-type EBS probe but not to a mutant probe (5'-CTGTAA-GA-3') (Figure 5C). To further confirm the binding of EIN3 to the promoter sequences of *HLS1*, we conducted a chromatin immunoprecipitation (ChIP) assay in 3-day-old etiolated *EIN3-FLAG/ein3 eil1* seedlings using anti-Flag antibody. *EIN3-FLAG/ein3 eil1* seedlings showed greatly increased binding of EIN3 to the EBS element compared with *ein3 eil1* seedlings. In contrast, a fragment from the 3' untranslated region (UTR) of *HLS1* did

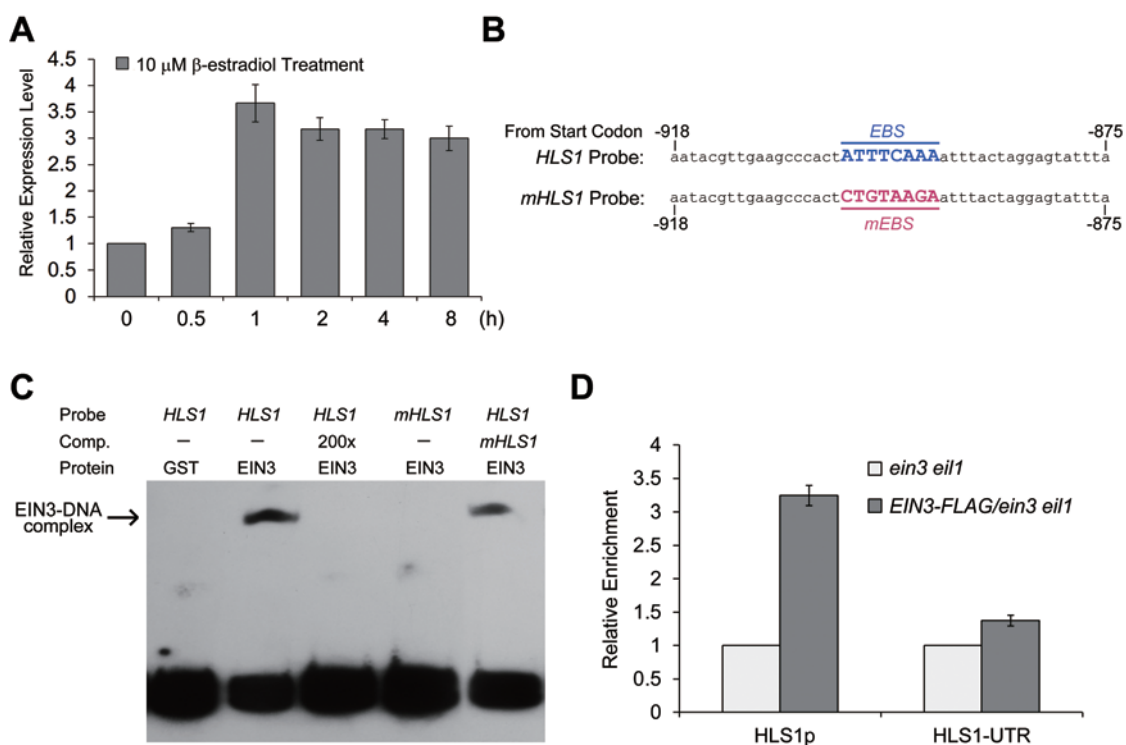


Figure 5 *HLS1* is a direct target gene of EIN3. **(A)** Rapid induction of *HLS1* expression in *iE/ein3 eil1* by estradiol. 3-day-old etiolated *iE/ein3 eil1* (a transgenic plant inducibly expressing EIN3-FLAG in the *ein3 eil1* background) seedlings grown on MS medium were treated with 10 μ M estradiol for indicated hours before the seedlings were collected for RNA extraction. Experiments were repeated three times with similar results. **(B)** Oligonucleotides used in the EMSA assays. The *HLS1* probe contains an EBS motif (underlined), which is a putative EBS. In the *mHLS1* probe, the EBS was mutated to *mEBS* (underlined). Number showed the location upstream of the start codon. **(C)** EMSA results showing *in vitro* binding of GST-EIN3 to the EBS motif in the promoter of *HLS1*. Protein-DNA complexes were detected when GST-EIN3 was incubated with labeled *HLS1* probe, and competition assays were conducted by adding 200-fold excessive unlabeled *HLS1* or *mHLS1* probe. GST-EIN3 was also incubated with labeled *mHLS1* probe and no protein-DNA complexes were observed. **(D)** ChIP assays indicating *in vivo* binding of EIN3 to the *HLS1* promoter sequence. Chromatin from *ein3 eil1* and transgenic plants constitutively expressing EIN3-FLAG in *ein3 eil1* was immunoprecipitated with an anti-FLAG antibody, and the quantification of the indicated DNA fragments in the precipitated chromatin was determined by quantitative real-time PCR. The amounts of DNA amplified from the *EIN3-FLAG/ein3 eil1* seedlings were normalized to that from *ein3 eil1* plants. The 3'-UTR fragment of *HLS1* was used as a negative control. The experiment was repeated three times with similar results.

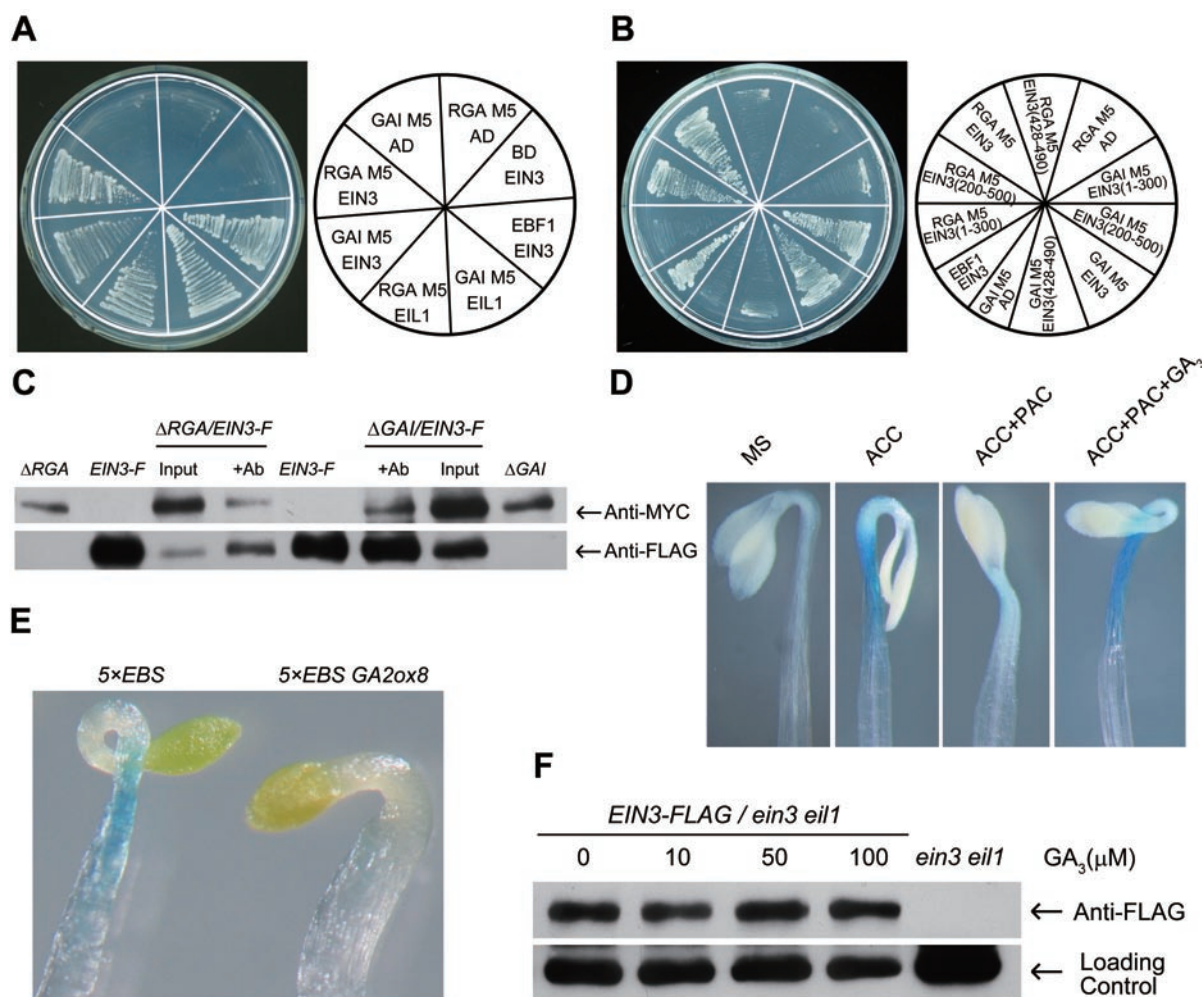


Figure 6 RGA/GAI interacts with EIN3/EIL1 and inhibits their function. **(A)** RGA/GAI interacted with EIN3/EIL1 in yeast cells. RGA M5 fragment (aa 209–587) and GAI M5 fragment (aa 158–533) were used in the yeast two-hybrid assays. EBF1/EIN3 interaction was used as a positive control. **(B)** EIN3 fragment (aa 200–500) was sufficient for the interactions with RGA and GAI in yeast cells. **(C)** Co-IP assays showing RGA/GAI association with EIN3 *in vivo*. *EIN3-FLAG/ein3 eil1* (*EIN3-F*) was crossed with *35S:TAP-RGA Δ 17* (Δ RGA) or *35S:TAP-GAI Δ 17* (Δ GAI), and F1 plants were used for Co-IP. Proteins were immunoprecipitated with anti-FLAG M2 agarose beads (+Ab) and detected with either anti-MYC or anti-FLAG antibodies. **(D)** PAC treatment inhibited the ACC-induced *5x EBS:GUS* expression. 3-day-old etiolated *5x EBS:GUS* seedlings grown on the indicated medium (ACC: 10 μ M, PAC: 1 μ M, and GA: 10 μ M) were used for GUS staining. **(E)** Expression of *35S:GA2ox8* in the *5x EBS:GUS* background decreased the ACC-induced GUS staining. 3-day-old etiolated *5x EBS-GUS* and *35S:GA2ox8 5x EBS-GUS* seedlings grown on 10 μ M ACC medium were used for GUS staining. **(F)** GA₃ treatment did not affect the protein level of EIN3 in *EIN3-FLAG/ein3 eil1*. 3-day-old etiolated seedlings grown on 0.2 μ M PAC were treated with indicated concentrations of GA₃ for 4 h before proteins were extracted for immunoblot assay with anti-FLAG antibody.

not show detectable enrichment by EIN3-FLAG (Figure 5D). Combining the results of *in vitro* EMSA and *in vivo* ChIP assays, we conclude that *HLS1* is a direct target gene of EIN3.

RGA/GAI physically interacts with EIN3/EIL1 and inhibits their function

DELLA proteins are central transcriptional repressors without DNA-binding ability [26]. It has been reported

that DELLAs associate with other transcription factors (like PIF3/4, ALC, and SCL3) to regulate downstream transcription and signal integration. Because DELLA proteins seemed to repress the expression of *HLS1*, which is a direct target gene of EIN3/EIL1, we speculated that DELLAs could also repress EIN3/EIL1 functions through protein-protein interaction. We first examined the interactions between EIN3/EIL1 and RGA/GAI in yeast cells. To avoid the strong self-activation of full-length

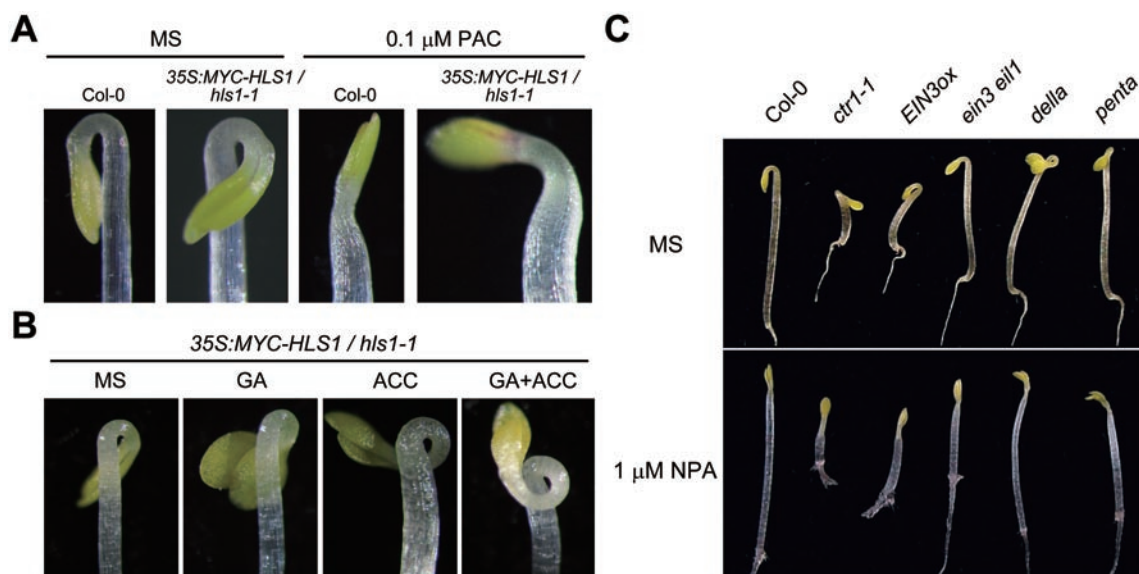


Figure 7 Blocking of auxin transport suppresses GA- and ethylene-induced hook curvature. **(A)** The hook phenotype of wild-type and *35S:MYC-HLS1/hls1* grown on MS medium supplemented with or without 0.1 μM PAC for 3 days. **(B)** The hook phenotype of *35S:MYC-HLS1/hls1* grown on MS medium supplemented with or without 10 μM ACC and/or 10 μM GA₃ for 3 days. **(C)** The hook phenotype of 3-day-old etiolated seedlings of indicated genotypes. Seedlings grown on MS medium and 1 μM NPA medium were shown.

RGA or GAI [26], we chose the N-terminal fragments of RGA (M5: aa 209-587) or GAI (M5: aa 158-533) in our assays, and found that these fragments of RGA/GAI and EIN3/EIL1 exhibited interactions (Figure 6A). We also tested the interactions between other members of these two families, and no interactions were observed except for the EIL2 interaction with RGA (Supplementary information, Figure S4A). We further found that an EIN3 fragment containing amino-acid residues 200-500 was sufficient for interacting with RGA/GAI (Figure 6B). Deletion of M5 from N-terminal or C-terminal disturbed the interaction (Supplementary information, Figure S4B). In addition, co-immunoprecipitation (Co-IP) studies using transgenic *EIN3-3FLAG/ein3 eil1* crossed to *35S:TAP-RGAΔ17* or *35S:TAP-GAIΔ17* further confirmed these interactions *in planta* (Figure 6C). Together, these results suggest that EIN3/EIL1/EIL2 are a new class of DELLA-associated transcription factors.

We next determined how RGA/GAI-EIN3/EIL1 interactions affect EIN3/EIL1 function. A *5×EBS:GUS* reporter line harboring five tandem repeats of *EBS* fused with the glucuronidase (*GUS*) reporter gene was previously used to monitor the function of EIN3/EIL1 [42]. We found that ACC evidently induced *5×EBS:GUS* expression in the hook/hypocotyl region, whereas the *GUS* expression was dramatically repressed by PAC treatment (Figure 6D). Moreover, GA₃ reversed the PAC effect by

enhancing the *GUS* expression. Consistently, overexpression of *GA2ox8* in the *5×EBS:GUS* reporter line led to a reduction of ACC-induced *GUS* expression (Figure 6E). These results suggest that accumulation of DELLA proteins acts to repress the function of EIN3/EIL1.

A principal mechanism for EIN3/EIL1 inactivation is to promote their protein degradation. We then tested whether GAs affect the protein level of EIN3, and found that GA₃ treatments did not markedly affect EIN3 protein abundance in the *EIN3-3FLAG/ein3 eil1* seedlings (Figure 6F). Since DELLA proteins interact with aa 200-500 of EIN3, a region responsible for DNA binding [43, 44], we speculate that DELLA/EIN3 interaction may repress the DNA-binding ability of EIN3.

Inhibiting auxin polar transport represses GA- and ethylene-induced exaggerated hook curvature

Our above data suggest that GAs promote hook curvature probably by inducing *HLS1* expression. To further assess the importance of *HLS1* regulation in GA-regulated hook development, we overexpressed MYC-tagged *HLS1* under the control of the constitutive 35S promoter in *hls1-1* background. As expected, the transgene expression rescued the hookless phenotype and the resulting plants exhibited exaggerated hook curvature even in the absence of ethylene or GAs (Figure 7A), supporting a role of *HLS1* regulation in PAC-regulated hook curva-

ture. We further found that *35S:MYC-HLS1/hls1* was partially resistant to PAC treatment compared with wild type (Figure 7A). However, *35S:MYC-HLS1/hls1* was still responsive to PAC, ACC, and ACC + GA₃ (Figure 7B), we thus examined the mRNA and protein levels of HLS1 in *35S:MYC-HLS1/hls1*, and found that neither mRNA level nor protein level of MYC-HLS1 was altered by ACC or GA₃ treatment (Supplementary information, Figure S5), indicating that there must be alternative pathways independent of *HLS1* transcription to mediate the effects of GAs and PAC on hook-curvature regulation. Previous studies have shown that treatment with NPA prevented the constitutive hook curvature of *HLS1ox* [8]. We next examined whether NPA also suppresses the exaggerated hook phenotypes caused by the activation of either ethylene or GA signaling. We found that treatment with NPA almost completely inhibited the apical hook-bending observed in *ctr1*, *EIN3ox*, *della*, and *penta* mutants (Figure 7C). This result indicates that auxin functions downstream of the ethylene and GA pathways to modulate hook curvature, thus raising a possibility that GAs and ethylene also influence auxin transport in addition to inducing *HLS1* expression. This conclusion is consistent with a recent report showing that GAs modulate auxin transport and response through PIN3 and PIN7 [45].

Discussion

Apical hook is a unique functional structure essential for plant germination in the soil that protects the cotyledons and apical meristemic stem from mechanical damage when protruding through the soil. Its formation is coordinately regulated by several plant hormones, including ethylene, auxin, GAs, jasmonate, and brassinosteroid [3, 16-18, 46-48]. The interplay between ethylene and auxin was extensively studied and several regulatory mechanisms have been revealed. However, the molecular details of the interactions among other hormones in the control of hook development remain largely unknown. In this study, we show that GAs and ethylene cooperatively regulate the hook curvature partly by inducing the gene expression of *HLS1*, a putative *N*-acetyltransferase essential for hook formation. We further demonstrate that EIN3/EIL1s are the integration node linking the two hormone pathways to directly activate *HLS1* transcription, and that GAs induce *HLS1* expression by relieving the inhibitory effect of DELLA proteins on EIN3/EIL1. In the meanwhile, GAs and ethylene also initiate the *HLS1*-independent pathways to regulate hook curvature, probably by modulating asymmetric auxin accumulation in the hook region (Figure 8).

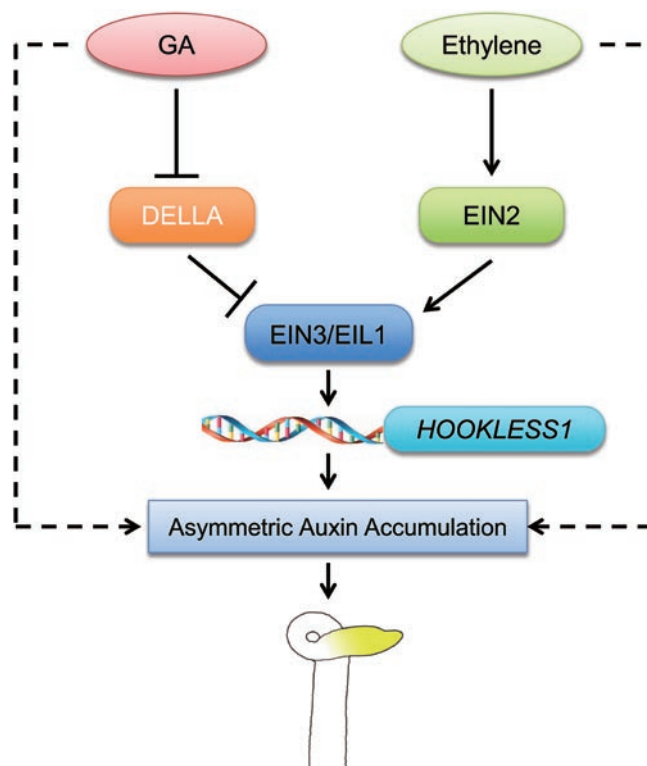


Figure 8 A proposed model of GAs and ethylene co-action in promoting the hook curvature. GAs and ethylene cooperatively regulate the hook curvature partly by inducing the gene expression of *HLS1*, a putative *N*-acetyltransferase essential for hook formation. EIN3/EIL1 are the integration node linking the two hormone pathways to directly activate the *HLS1* transcription, in which ethylene stabilizes EIN3/EIL1, while GAs relieve the repression of DELLA proteins on EIN3/EIL1. Meanwhile, GAs and ethylene also initiate the *HLS1*-independent pathways to regulate hook curvature, probably by modulating asymmetric auxin accumulation in the hook region. The solid lines indicate proved regulations, whereas the dotted lines indicate proposed regulations.

Treatment of PAC, a GA-biosynthesis inhibitor, was previously reported to radically repress ethylene-induced hook curvature. Our study provides convincing evidence to indicate that PAC/GA-mediated regulation of hook curvature is partially dependent on the action of EIN3/EIL1, two ethylene-stabilized transcription factors. First, we found that overexpression of EIN3 (*EIN3ox*) resulted in partial resistance to either PAC treatment (Figure 1D) or GA deficiency (Figure 1C) in terms of hook curvature. *EIN3ox* also exhibited exaggerated hook bending upon GA₃ treatment, reminiscent of wild-type seedlings treated with ethylene plus GA₃ (Figure 1A and 1B). Second, loss of EIN3/EIL1 function remarkably suppressed the constitutive hook-bending phenotype of *della* mutant

(Figure 2). Similarly, *della* mutant was also suppressed by *ein2*, a mutant with no EIN3 or EIL1 protein accumulation. Third, we showed that *HLS1* is a direct target gene of EIN3 (Figure 5), and further found that GA₃-induced *HLS1* expression was dependent on EIN3/EIL1 (Figure 4). Lastly, we revealed that both EIN3 and EIL1 physically interacted with DELLA proteins, and such association led to the inhibition of EIN3/EIL1-regulated gene expression (Figure 6).

On the other hand, it is notable that GA control of hook curvature is not solely dependent on the EIN3/EIL1 pathway, as the *ein3 eil1* mutant was still responsive to PAC treatment (Figure 1D). In addition, *ein3 eil1* suppressed the constitutive hook phenotype of *della* to a half way (Figure 2B), whereas NPA treatment almost completely blocked its hook formation (Figure 7C). Therefore, DELLA proteins might direct an additional pathway independent of EIN3/EIL1 to modulate auxin production, transport, or signaling in hook development. DELLA proteins were reported to interact with PIF3/4 transcription factors and repress their activity. Interestingly, loss of multiple PIF functions also resulted in a hookless phenotype [49], suggesting an important role of PIF proteins in maintaining hook curvature. Together, these results indicate that GA-increased hook curvature is mediated through the removal of DELLA proteins, and DELLA accumulation reduces hook curvature by repressing the functions of EIN3/EIL1, and probably PIFs as well, to modulate the downstream auxin responses.

Previous studies have demonstrated that *HLS1* is a key mediator of multiple signals, including ethylene, auxin, and light, in the control of hook curvature [3]. Our results further support the central role of *HLS1* in mediating hormone signals (GAs and ethylene) in hook development. We first showed that the *hls1* mutant was completely insensitive to ethylene and/or GA₃ treatment (Figure 3A), and the mutation also suppressed the exaggerated hook phenotype of the *della* mutant (Figure 3B). Furthermore, we found that both GA₃ and ethylene induced *HLS1* expression (Figure 4), and that EIN3 appears to directly bind to the promoter of *HLS1* (Figure 5). Thus, induction of *HLS1* expression represents a principal mechanism of GA- and ethylene-mediated regulation of hook development. Nevertheless, we also observed that plants with constitutive overexpression of *HLS1* in the *hls1*-mutant background (*HLS1ox/hls1*) were still responsive to ethylene, PAC, GA₃, or their combined treatment (Figure 7A and 7B). Given that the transcript or protein level of MYC-*HLS1* was not affected by ACC and GA₃ (Supplementary information, Figure S5), these results suggest the existence of the alternative GA and ethylene signaling pathways independent of *HLS1* induc-

tion (Figure 8). Our results and previous studies showed that blocking of auxin transport by NPA represses the constitutive hook curvature of *HLS1ox*, *ctr1*, *EIN3ox*, and *della* (Figure 7C), suggesting that modulation of auxin distribution is a common downstream event in the GA- and ethylene-mediated regulation of hook formation. Ethylene was also recently reported to regulate hook curvature by influencing auxin influx and efflux carriers at transcriptional and posttranscriptional levels [16, 17]. It is thus possible that GAs might alter the expression level or activity of auxin influx and/or efflux transporters as well.

Accumulating evidence has demonstrated that DELLA proteins control a wide variety of plant growth and stress responses. With no intrinsic DNA-binding abilities, DELLA proteins were thought to function as transcriptional repressors that interact with various transcription factors and repress their activity [24, 25]. To date, DELLA proteins have been reported to interact with PIF3/4, ALC, and SCL3 transcription factors to mediate the effects of GA on hypocotyl elongation, valve margin development, and root development, respectively. In this study, we have identified EIN3/EIL1 as a new class of DELLA-associated transcription factors. Interestingly, only RGA and GAI but not RGL proteins interacted with EIN3/EIL1, suggesting the functional diversity of DELLA proteins in different processes. As GA₃ treatment had no effect on EIN3 protein stability, plus the observation that DELLA proteins interacted with the DNA-binding domains of EIN3/EIL1, we speculate that DELLA association could block the binding of EIN3/EIL1 to their target promoters. Further experiments would help verify this possibility in the future. Since GAs and ethylene coordinately regulate a number of plant growth and development processes, such as seed germination, stem elongation, and stress tolerance, it would be interesting to investigate whether the DELLA-EIN3/EIL1 interaction also plays a part in the coordinated action of the two hormones in these processes.

Materials and Methods

Plant materials and growth conditions

ein2-5 [50], *ein3-1 eil1-1* [32], *ctr1-1* [14], *eto1-2* [12], *hls1-1* [12], *penta* (*gal-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) [51], and *della* (*rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1*) [27] mutants and EIN3ox (35S:EIN3) [34], *iE/qm* [33], *5×EBS:GUS* [42], *35S:TAP-RGAΔ17(ΔRGA)* [27], *35S:TAP-GAIΔ17(ΔGAI)* [27], and *35S:GA2ox8* [35] transgenic plants were described previously.

della hls1, *della ein2*, and *della ein3 eil1* multiple mutants were generated by genetic crosses, and putative etiolated *della hls1*, *della ein2*, and *della ein3 eil1* homozygous lines were initially identified by comparison with the parental phenotype on the MS medium supplemented with 10 μM PAC plus 10 μM ACC, then

confirmed through PCR-based genotyping. The primer pairs for identifying *ein2*, *ein3*, *eil1*, and DELLA-related mutants were as described previously [33, 52].

Estradiol-inducible *EIN3-FLAG* in the *ein3 eil1* background (*iE/ein3 eil1*) was generated by crossing *iE/qm* (estradiol-inducible *EIN3-FLAG* in the *ein3 eil1 ebf1 ebf2* background) into *ein3 eil1* background and then screened on 10 μ M estradiol in the dark. Those with longer roots compared with *iE/qm* were further genotyped with the *ebf1* and *ebf2* sites. The primer pairs for identifying *ebf1* and *ebf2* were as described previously [33].

To construct transgenic plants constitutively expressing *EIN3-3FLAG*, *EIN3* cDNA was PCR amplified from reverse transcription product with primers 5'-AGGTTCTGAAGAACCATATGGATACATCTTG-3' and 5'-AAACTCGAGATGATGTTTAATGAGATGGGAATG-3', digested with *XhoI* and *Csp45I*, inserted into a pUC19-derived plasmid containing triple FLAG-tag, and then digested with *KpnI* and *PstI*, inserted into a pCambia1300 vector. To construct transgenic plants constitutively expressing *MYC-HLS1*, *HLS1* cDNA was PCR amplified from reverse transcription product with primers 5'-TCCCCCGGGACATGACGGTGGTTAGAGAGTAC-3' and 5'-GCGGAGCTCGTAGGAGATATTACCTCTTACC3-', digested with *SmaI* and *SacI*, inserted into a pBA-MYC vector. All constructs were verified by DNA sequencing analysis. The resultant constructs were introduced into *Agrobacterium* strain C58, and subsequently the 35S:*EIN3-3FLAG* construct was transformed into *ein3-1 eil1-1* plants and the 35S:*6MYC-HLS1* constructs were transformed into *hls1-1* by vacuum infiltration [53].

Surface-sterilized seeds were plated on MS medium (4.3 g/l MS salts, 1% sucrose, pH 5.7-5.8, and 8 g/l agar) and imbibed for 4 days at 4 °C. For hook phenotype, the plates were wrapped in foil and kept in a 22 °C incubator before the phenotypes of seedlings were analyzed. For adult plant phenotypic analysis, light-grown seedlings from plates were transferred to soil and grown to maturity at 22 °C under a 16-h light/8-h dark cycle.

Chemical solutions

All chemicals were from Sigma-Aldrich, unless specified otherwise, and prepared as stock solutions. DMSO was used to dissolve NPA (100 μ M), MG132 (50 mM), and β -estradiol (10 mM). 100% ethanol was used to dissolve GA₃ (71.43 mM) and PAC (10 mM). ACC (10 mM) and AVG (10 mM) were dissolved in water. Final concentrations were 1 μ M NPA, 10 μ M ACC, and 10 μ M AVG for germination, 50 μ M MG132 for Co-IP, and 100 μ M GA₃ and 100 μ M ACC for seedling treatment.

Immunoblot assays

For EIN3-3FLAG western blot, 3-day-old dark-grown *EIN3:3FLAG/ein3 eil1* transgenic seedlings were transferred to MS liquid medium with different concentrations of GA₃ treatment for 4 h and protein was extracted in the buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% Triton X-100, 1 mM PMSF, and 1 \times protease inhibitor cocktail). EIN3-3FLAG fusion proteins were determined by immunoblot using anti-FLAG antibody (Sigma-Aldrich) diluted in 5 000 folds.

Real-time quantitative reverse transcription PCR analysis of gene expression

Total RNA was prepared using the TRIzol Reagent (Invitrogen, Inc.). In all, 2 μ g total RNA treated with DNase I (Promega, Inc.)

was added in a 20 μ l reverse transcription reaction using the M-MLV Reverse Transcription System (Promega, Inc.). Real-time PCR was performed on the LightCycler 480 Real-Time PCR System (Roche) using SYBR Green Mix (Takara, Inc.) and the specific primers of *HLS1* (5'-CACGGTTATCAAGTTAGAGC-3' and 5'-GAAAGTCCCAAGCGAGA-3'). The expression level was normalized to β -tubulin (5'-GAGCCTTACAACGCTACTCTGTCT-GTC-3' and 5'-ACACCAGACATAGTAGCAGAAATCAAG-3') control. Experiments were biologically repeated at least two times.

ChIP assay

ChIP was performed as described [43] using 3-day-old dark-grown seedlings on MS medium treated with 100 μ M ACC. Mouse anti-FLAG M2-agarose beads (Sigma-Aldrich) were used for IP. The enrichment of DNA fragments was determined by quantitative real-time PCR using primer pairs HLS1-p-chip: 5'-CCACATCAATGCTCGTCTTA-3' and HLS1-pr-chip: 5'-CAGTGGCGCTATCTATTTTC-3', and primer pairs: HLS1-3' UTR-CHIP: 5'-GGCGTTCCATTTTGTAGAC-3', and HLS1-3' UTR-CHIP: 5'-GGAGATATTACCTCTTACCAC-3' were used as a negative control.

Protein expression

GST-EIN3 (aa 1-314) constructs were transformed into BL21 (DE3)-competent cells. Protein expression was induced by 0.1 mM isopropyl-beta-thiogalactopyranoside and fusion proteins were prepared following the manufacturer's instructions.

EMSA

Oligonucleotide probes (HLS1: 5'-AATACGTTGAAGC-CCACTATTTCAAATTTACTAGGAGTATTTA-3' and 5'-TAAATACTCCTAGTAAATTTTGAAATAGTGGGCT-TCAACGTATT-3'; mHLS1: 5'-AATACGTTGAAGCCCA-CTCTGTAAGAATTTACTAGGAGTATT-3' and 5'-AATACTC-CTAGTAAATTTCTTACAGAGTGGGCTTCAACGTATT-3') were synthesized and labeled with a Biotin 3'-end DNA Labeling Kit. EMSA was performed using a LightShift Chemiluminescent EMSA Kit. In total, 20 fmol of labeled probes were incubated in 1 \times binding buffer, 2.5% glycerol, 50 mM KCl, 5 mM MgCl₂, and 10 mM EDTA with or without proteins at room temperature for 20 min. For non-labeled probe competition, the amount of HLS1 and mHLS1 competitors is 200-fold more than that of labeled probe.

Yeast two-hybrid assays

The coding sequences of *RGA M5* (209-587), *RGA* (256-587), *RGA* (323-587), *RGA* (209-372), *RGA* (209-294), *GAI M5* (158-533), *RGL1 M5*, *RGL2 M5*, and *RGL3 M5* were amplified from wild-type cDNA and cloned into pGBKT7 vectors, while the coding sequences of *EBF1*, *EIN3*, *EIN3* (200-500), *EIN3* (1-300), *EIL1*, *EIL2*, *EIL3*, *EIL4*, and *EIL5* were cloned into pGADT7 vectors and transformed into yeast strain AH109 following the Matchmaker user's manual protocol (Clontech). Transformed yeast cells were streaked onto SD (-Leu/-His/-Ade/-Trp) medium (Clontech) and grown at 30 °C for 4 days. The white colonies represented interactions. All the primers used in this study are summarized in Supplementary information, Table S1.

EIN3-3FLAG and Δ *RGA-MYC*/ Δ *GAI-MYC* Co-IP assays

Co-IP assays of the EIN3-RGA/GAI proteins were performed

with 7-day-old F1 seedlings derived from genetic crosses between *EIN3-3FLAG/ein3 eil1* (EIN3-F) and Δ *RGAMYC/ΔGAIMYC* grown under white light. Seedlings were treated with 100 μM ACC for 4 h prior to protein extraction. Immunoprecipitation of the EIN3-3FLAG protein was performed at 4 °C for at least 6 h, using anti-FLAG M2-agarose beads (Sigma-Aldrich) in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% Triton X-100, 1 mM PMSF, and 1× protease inhibitor cocktail (Roche). EIN3-3FLAG and Δ *RGAMYC/ΔGAIMYC* detection were performed with anti-FLAG and anti-MYC antibodies, respectively.

GUS staining

Seedlings were incubated in GUS staining buffer (100 mM Na₃PO₄, pH 7.0, 1 mM EDTA, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 1% Triton X-100, and 1 mg/ml X-Gluc) and then washed in washing solution (100 mM Na₃PO₄ and 1 mM EDTA) before images were taken.

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(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)